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16. ABSTRACT
Recent work in our laboratory has shown the presence of many neuron-specific phosphoproteins in the mammalian nervous system [6, 9, 12-15]. Two of these proteins, Protein III and Synapsin I, are specifically associated with synaptic vesicles in neurons throughout the brain. Protein III consists of two polypeptides, Protein IIIa (M_r 74 kD) and Protein IIIb (M_r 55 kD). These two polypeptides have similar chemical immunological and biological properties ([6]; Browning, Huang, and Greengard, manuscript in preparation), and they seem to be closely related to Synapsin I which also consists of two polypeptides, Ia (M_r 86 kD) and Ib (M_r 80 kD). Protein III and Synapsin I may play roles in synaptic function as their states of phosphorylation are increased by agents that depolarize neuronal membranes, by 8-bromoadenosine 3':5'-monophosphate (cAMP), and by several neurotransmitters, including dopamine, serotonin and norepinephrine [4, 5, 9, 11]. In the present study, we investigated Protein III and Synapsin I in human brain tissue obtained at autopsy from normal individuals and from individuals suffering from a variety of diseases affecting the central nervous system. We report here data indicating variant forms of Protein III in human brain tissue.

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Protein III, a Neuron-Specific Phosphoprotein: Variant Forms Found in Human Brain[1]

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PERDAHL, E., W. C.-S. WU, M. D. BROWNING, B. WINBLAD AND P. GREENGARD. *Protein III, a neuron-specific phosphoprotein: Variant forms found in human brain.* NEUROBEHAV TOXICOL TERATOL 6(6) 425–431, 1984.—Protein III is a neuron-specific phosphoprotein which consists of two polypeptides, IIIa (M_r 74 kD) and IIIb (M_r 55 kD). This phosphoprotein has previously been shown to be associated with synaptic vesicles. In the present investigation, we have examined Protein III in human brain tissue. In contrast to observations in rat brain, where only one form of Protein IIIb (M_r 55 kD) has been found, in human brain tissue three variants of Protein IIIb, designated Protein $IIIb_1$ (M_r 55 kD), Protein $IIIb_2$ (M_r 57 kD) and Protein $IIIb_3$ (M_r 59 kD), were observed by both biochemical and immunochemical assays. Protein IIIa from human brain also exhibited three variants in electrophoretic mobility. Peptide maps of Proteins IIIa and IIIb revealed that the differences in electrophoretic mobility of the variants of these proteins were preserved in variants (M_r 18kD, M_r 20kD, and M_r 22kD) of a smaller peptide fragment. These variant forms of Protein III were studied in brains from individuals without any history of neurological or psychiatric illness, as well as from individuals who had suffered from one of several types of neuropathological conditions, such as chronic alcoholism, Alzheimer's disease, multi-infarct dementia, and Parkinson's disease. Some differences were observed in the distribution of variants among the various clinical categories.

Phosphoprotein Brain Human Vesicles Polypeptide

RECENT work in our laboratory has shown the presence of many neuron-specific phosphoproteins in the mammalian nervous system [6, 9, 12–15]. Two of these proteins, Protein III and Synapsin I, are specifically associated with synaptic vesicles in neurons throughout the brain. Protein III consists of two polypeptides, Protein IIIa (M_r 74 kD) and Protein IIIb (M_r 55 kD). These two polypeptides have similar chemical, immunological and biological properties ([6]; Browning, Huang, and Greengard, manuscript in preparation), and they seem to be closely related to Synapsin I which also consists of two polypeptides, Ia (M_r 86 kD) and Ib (M_r 80 kD). Protein III and Synapsin I may play roles in synaptic function as their states of phosphorylation are increased by agents that depolarize neuronal membranes, by 8-bromoadenosine 3':5'-monophosphate (cAMP), and by several neurotransmitters, including dopamine, serotonin and norepinephrine [4, 5, 9, 11]. In the present study, we investigated Protein III and Synapsin I in human brain tissue obtained at autopsy from normal individuals and from individuals suffering from a variety of diseases affecting the central nervous system. We report here data indicating variant forms of Protein III in human brain tissue.

METHOD

Materials

[γ-^{32}P]ATP (specific activity 2,900 Ci/mmol) and ^{125}I-Protein A (specific activity 70–100 μCi/μg) were obtained from New England Nuclear, Boston, MA, USA; Nonidet-P 40, Triton X-100, phenylmethylsulfonyl fluoride, Pepstatin A and 8-bromoadenosine 3':5'-monophosphate were obtained from Sigma, St. Louis, MO, USA; nitrocellulose filters were obtained from Schleicher and Schuell, Keane, NH, USA; Staphylococcus aureus V_8 protease was obtained from Miles Laboratories, Inc., Elkhart, IN, USA; Protein A-bearing Staphylococcus aureus cells (Pansorbin) were obtained from Calbiochem-Behring, La Jolla, CA, USA; highly purified catalytic subunit of cAMP-dependent protein kinase was kindly provided by Dr. A. C. Nairn of this laboratory.

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ABBREVIATIONS

M_r	relative molecular weight	SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
kD	kilodalton	SDAT	senile dementia of Alzheimer type
cAMP	3′:5′-adenosine monophosphate	MID	multi-infarct dementia
ATP	adenosine triphosphate	IA	intoxicated alcoholic
EDTA	ethylene-diaminetetraacetic acid	NIA	non-intoxicated alcoholic
EGTA	ethylene-glycoltetraacetic acid		

TABLE 1

DATA FROM SERIES OF STUDIES ON ALCOHOLIC SUBJECTS

	n	Age Years	Sex M/F	Brain-weight g	Post-mortem time hr	Blood ethanol mg/100 ml
Controls	17	56.8 ± 18.4	15/2	1449 ± 101	54.5 ± 22.8	negative
IA	17	45.0 ± 12.4	14/3	1488 ± 110	61.9 ± 33.7	272.8 ± 98.9 (h) 275.9 ± 150.0 (v)
NIA	12	55.4 ± 11.4	9/3	1468 ± 182	49.9 ± 22.5	negative

Data are presented as means ± standard deviations.
(h), heart blood; (v) venous blood from the femoral vein.

Brain Tissue

In one series of studies, brains were removed at complete autopsies in the Department of Forensic Medicine, University of Umeå, Sweden, and 9 brain regions (frontal cortex, cingulate gyrus, hippocampus, caudate nucleus, putamen, hypothalamus, thalamus, substantia nigra and cerebellum) were immediately dissected, put into airtight packages and frozen at −80°C until assayed. Blood samples from the heart and the femoral vein, and urine, were collected for analysis of ethanol concentration. A piece of liver was dissected and subjected to microscopy for determination of liver steatosis and/or cirrhosis.

Forty-six cases were investigated. Twenty-nine of these cases were known to have a history of alcohol abuse for more than 5 years (based on police reports, hospital records, and interviews with family and friends). Liver steatosis and/or cirrhosis was present in all 29 cases. Seventeen of the alcoholics (14 men, 3 women, mean age 45 years, range 30–65 years) were intoxicated (defined as blood ethanol levels >50 mg/100 ml) at the time of death with a blood ethanol level ranging from 90 mg/100 ml to 420 mg/100 ml (intoxicated alcoholics=IA). Twelve alcoholics (9 men, 3 women, mean age 55 years, range 33–73 years) were sober (no detectable blood ethanol) at the time of death (non-intoxicated alcoholics=NIA). Seventeen control cases (15 men, 2 women, mean age 56 years, range 19–90 years) were without any history of neurological or psychiatric illness or any drug abuse.

Causes of death among the alcoholics were pneumonia (5 cases), intoxication with ethanol (in some cases combined with methanol or other organic solvents) (10 cases), suicide (3 cases), inhalation of stomach content into the lungs (4 cases), circulatory failure (6 cases) or subarachnoidal bleeding (1 case). Causes of death among the controls were accident (3 cases), suicide (1 case), asthma attack (1 case) and circulatory failure, including myocardial infarction (12 cases). Age, brain weight and post-mortem time are presented in Table 1.

In a second series of studies, 7 brain regions (frontal cortex, parietal cortex, hippocampus, caudate nucleus, putamen, cingulate gyrus and mesencephalon) were dissected and frozen as described above, during autopsies of 38 individuals in the Department of Pathology, University of Umeå, Sweden. Routine histopathological examination (hematoxylin-eosin staining) was performed on frontal cortex, parietal cortex and hippocampus. Frozen sections from these regions were also silver-stained according to von Braunmühl [2] for estimation of senile plaques and neurofibrillary tangles. Based on clinical (DSM III) and histopathological findings, 13 individuals were classified as suffering from senile dementia of Alzheimer's type (SDAT), 9 individuals were classified as suffering from multi-infarct dementia (MID), 3 were classified as suffering from Parkinson's disease (2 with dementia and 1 without dementia) and 2 were classified as suffering from dementia of unknown cause (dementia NUD). Eleven age-matched individuals without any history of neurological or psychiatric disease served as controls. Age, brain weight and post-mortem time are presented in Table 2.

Phosphorylation of Acid-Soluble Phosphoproteins

Acid extraction and back-phosphorylation were carried out as described by Walaas *et al.* [14]. Briefly, 5 volumes of cold zinc acetate were added to homogenates in 10 mM Tris (pH 7.4), 2 mM EDTA (or 25 mM Hepes pH 7.4, 2 mM EDTA) and precipitated proteins were collected by centrifugation at 4,000×g for 15 min. The pellets were resuspended in 1 vol of cold 10 mM citrate-phosphate buffer (pH 2.8–3.0) containing 0.1% Triton X-100 (non-ionic detergent) and 2

TABLE 2

DATA FROM SERIES OF STUDIES ON DEMENTED SUBJECTS

	n	Age years	Sex M/F	Brain-weight g	Post-mortem time hr
Controls	11	84.0 ± 8.8	5/6	1307 ± 121	33.3 ± 10.0
SDAT	13	78.3 ± 8.5	10/3	1229 ± 386	27.0 ± 15.0
MID	9	80.0 ± 7.3	4/5	1321 ± 119	43.2 ± 17.4
Parkinsonian	3	77.7 ± 5.1	3/0	1433 ± 58	24.0 ± 17.5
Dem NUD	2	42/75	2/0	1180/1200	19.3/67.4

Data are presented as means ± standard deviations.
In the group with less than 3 cases, individual values are presented.

μg/ml of the protease inhibitor Pepstatin A. The acid-insoluble proteins were removed by centrifugation at 27,000×g for 15 min. The supernatants were neutralized (final pH 6.0–6.5) by adding 1/10 vol of 0.5 M Na_2HPO_4 and centrifuged at 27,000×g for 15 min, to remove insoluble material. The neutralized extracts were kept on ice while protein concentration was determined according to Lowry *et al.* [8].

After being adjusted to equal protein concentration (10–50 μg), the neutralized extracts were subjected to back-phosphorylation in a medium (final volume 0.1 ml) containing (final concentration) 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 25 nM catalytic subunit of cAMP-dependent protein kinase, and 2 μM [γ-^{32}P]ATP. The reaction was carried out for 30 min at 30°C. It was terminated by addition of SDS-containing stop-solution and the mixture boiled for 3 min. The samples were then subjected to discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [7]. The gels were fixed, stained, destained, dried and subjected to autoradiography (Kodak XR-5 film) as described by Ueda and Greengard [12].

A number of experiments were carried out in which samples from different individuals (controls, IA, NIA), and from fresh rat brain, were mixed and compared with the individual samples themselves. In all cases, the results were additive, suggesting that differences in the M_r values for Protein III were not due to either variable protease activity, salt concentration or other factors in the samples. In addition, several different homogenization conditions were tested (1% SDS; 2–25 mM Tris-HCl, pH 7.4; 2–50 mM Hepes, pH 5.5–8.0; 5 mM Zn-acetate; 0.32 M sucrose; H_2o), and none of these changed the results.

One-Dimensional Peptide Maps

One-dimensional peptide mapping was performed essentially as described by Cleveland *et al.* [3]. Following electrophoresis in an 8% SDS-polyacrylamide gel, the phosphorylated Protein IIIa or IIIb bands were localized by autoradiography and cut from the dried gel. The gel slices were soaked in 2 ml of 125 mM Tris (pH 6.7), 0.1% SDS, for 20 min and then placed into the sample wells of a second polyacrylamide gel. Each gel slice was overlaid with 5–10 μg of Staphylococcus aureus V_8 protease in 50 μl of 125 mM Tris (pH 6.7), 0.1% SDS, 10% glycerol. Following electrophoresis the ^{32}P-labeled peptide fragments of Protein IIIa and IIIb were identified by autoradiography.

Immunolabeling of Protein III

Neutralized extracts were subjected to SDS-PAGE and then transfered to nitrocellulose filters (0.2 μm pore size) in a Hoefer Scientific Instruments' transfer tank as described by Towbin *et al.* [10]. The filters were then overlaid with antibodies against Protein III and ^{125}I-Protein A as described by Adair *et al.* [1] with the following modifications: After transfer to the filter had been completed, the filter was washed in 50 ml of incubation-buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2% sodium azide) containing 5% (w/v) bovine serum albumin (BSA) for 45 min at 37°C; BSA (1% w/v final concentration) was also added to the ^{125}I-Protein A incubation-buffer; in the final wash, 1% Nonidet-P 40 (a non-ionic detergent) was added to the buffer and the filter was washed for 30 min in the detergent. These changes helped reduce non-specific binding to the nitrocellulose filter.

RESULTS

Variants of Protein III in Human Brain Tissue

Phosphorylated proteins. Acid extracts of the frontal cortex were phosphorylated *in vitro* and then subjected to SDS-polyacrylamide gel electrophoresis. Autoradiography of the dried gels revealed that there were three variant forms of Protein IIIb and these were designated Protein $IIIb_1$ (M_r 55 kD, which co-migrated with rat Protein IIIb), Protein $IIIb_2$ (M_r 57 kD) and Protein $IIIb_3$ (M_r 59 kD) (Fig. 1). Some samples showed only a single variant, and others showed two of the three variant forms of Protein IIIb. Protein IIIa also exhibited three variant forms, but the two variants with slower electrophoretic mobilities ($IIIa_2$ and $IIIa_3$) could not be reliably analyzed because their electrophoretic mobilities were similar to that of the prominent phosphoprotein Synapsin Ib. Neither Synapsin Ia nor Ib showed any change in M_r in any of the subjects investigated.

Identical variants were observed in all other brain regions investigated (cingulate gyrus, hippocampus, caudate nucleus, putamen, hypothalamus, thalamus, substantia nigra, cerebellum, parietal cortex and mesencephalon). Results similar to those observed in acid extracts were obtained when phosphoproteins were assayed by endogenous phosphorylation using the method either of Walaas *et al.* [14] or of Wu *et al.* [16].

One-dimensional peptide mapping of Protein III. The ^{32}P-labeled Protein IIIa and Protein IIIb bands were cut from a dried gel and subjected to limited proteolysis in a second

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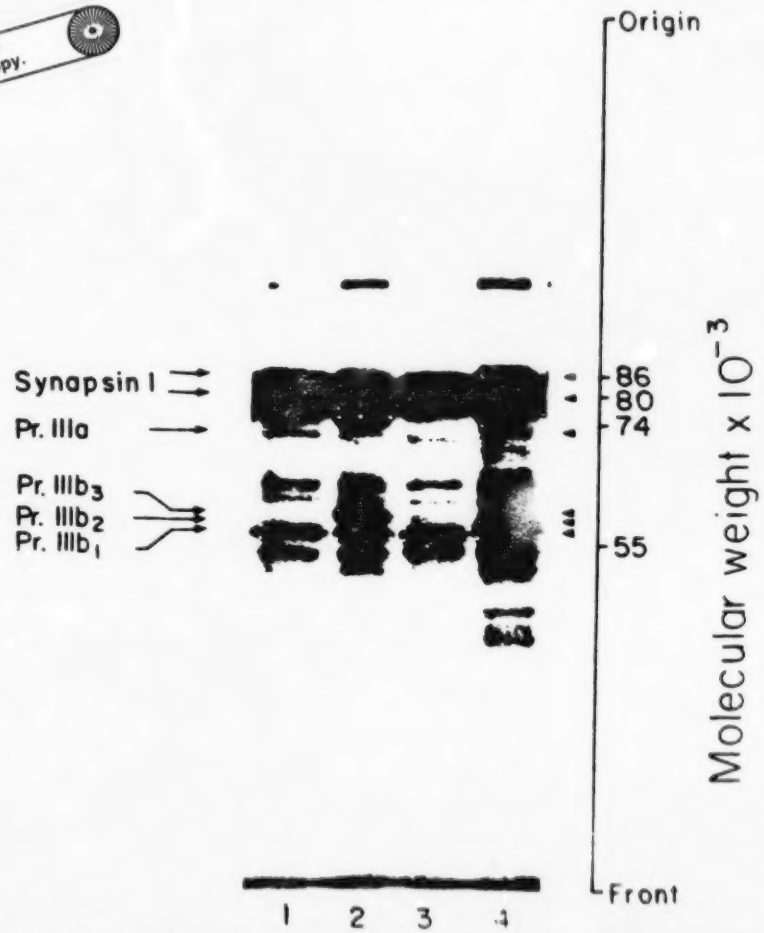

FIG. 1. Autoradiogram showing ^{32}P-incorporation into acid-soluble phosphoproteins from human frontal cortex. Synapsin I, Protein IIIa and the three variant forms of Protein IIIb are indicated by arrows and arrowheads. The variants present in the samples analysed are: lane 1, Protein $IIIb_1$ (control); lane 2, Proteins $IIIb_1$ and $IIIb_3$ (NIA); lane 3, Protein $IIIb_1$ (IA); lane 4, Proteins $IIIb_1$ and $IIIb_2$ (NIA).

polyacrylamide gel. Protein IIIb variants $IIIb_1$, $IIIb_2$, and $IIIb_3$ yielded fragments of 18kD, 20kD, and 22kD, respectively. Thus the differences in M_r values among the three variants of Protein IIIb were retained in the smaller phosphopeptide fragments obtained from the variants. Similarly, Protein IIIa variants $IIIa_1$, $IIIa_2$, and $IIIa_3$ yielded fragments of 18kD, 20kD, and 22kD, respectively. In addition, in each brain sample, the fragments observed for Protein IIIa corresponded to those observed for Protein IIIb (compare Fig. 2A and B).

In previous studies, one-dimensional peptide maps of rat Proteins IIIa and IIIb yielded a single 18kD phosphopeptide. Thus the human variants of Protein III ($IIIa_1$ and $IIIb_1$) which comigrated with rat Protein III yielded fragments in 1-dimensional phosphopeptide maps that comigrated with the phosphopeptide fragments obtained from rat Protein III. It should be noted that no shift in M_r was seen in the 9kD fragment obtained from any of the variants of Protein III by this limited proteolysis procedure.

Immunolabeling of Protein III. Antibodies which were specific for both Protein III and Synapsin I were used to immunolabel acid extracts. The antibodies recognized Synapsin I and the three variants of Protein IIIb (Fig. 3). In each sample, the variant(s) of Protein IIIb that were detected by immunolabeling co-migrated with the variant(s) identified by phosphorylation. The level of Protein IIIa was below the amount necessary for accurate analysis of the variants. In another set of experiments, it was found that immunoprecipitation of phosphorylated acid extracts, followed by SDS-polyacrylamide gel electrophoresis, gave results analogous to those obtained by immunolabeling (data not shown).

Clinical Studies

The variants of Proteins IIIa and IIIb were analysed in human brain tissue from normal individuals and from individuals who had suffered from a variety of neuropathological conditions. In 15 out or 17 control subjects in one group of

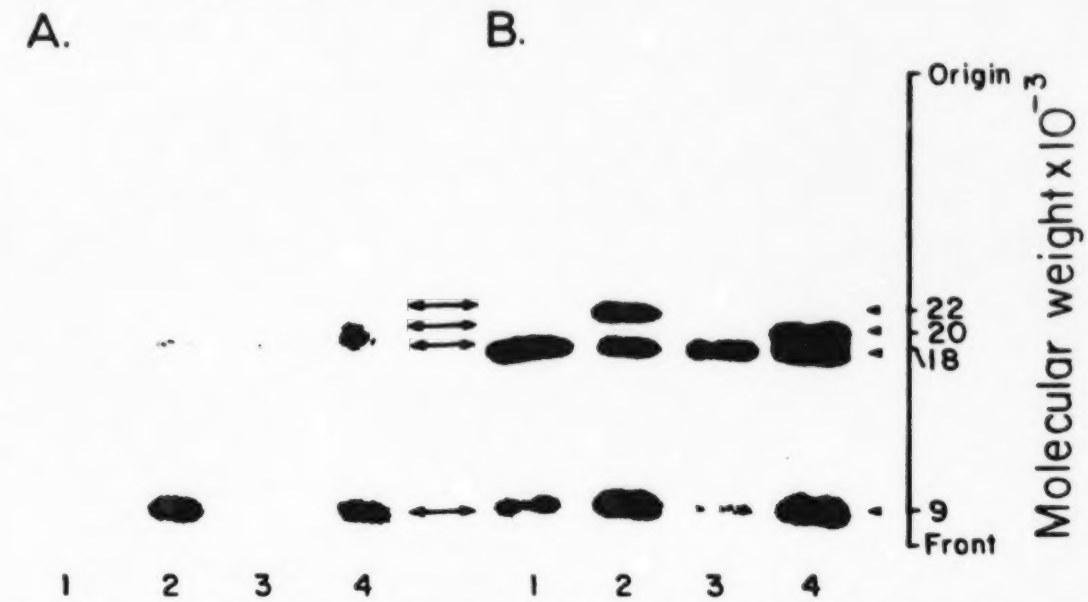

FIG. 2. Autoradiograms showing one-dimensional peptide maps of Proteins IIIa and IIIb from human frontal cortex. The Protein IIIa and IIIb bands shown in Fig. 1 were subjected to limited proteolysis by Staphylococcus aureus V_8 protease. The resultant peptide fragments of the variants are indicated by arrows and arrowheads. A, peptide fragments derived from Protein IIIa; B, peptide fragments derived from Protein IIIb.

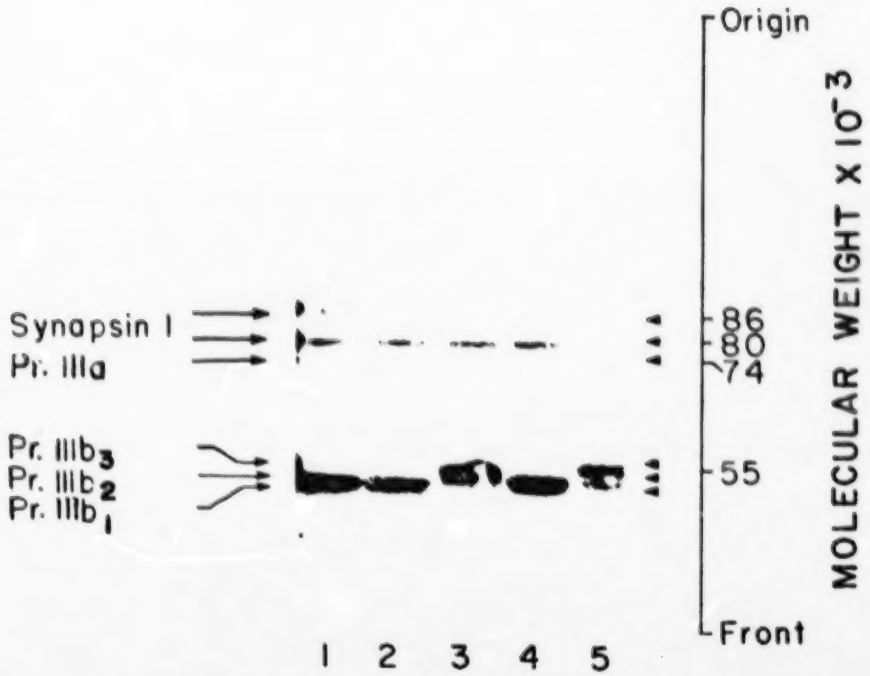

FIG. 3. Autoradiogram showing immunolabeling of Protein III in extracts from frontal cortex. Synapsin I and the three variant forms of Protein IIIb are indicated by arrows and arrowheads. The faint labeling in the region below Synapsin I (indicated by arrowhead) is due to immunolabeling of Protein IIIa. The variants present in the samples analysed are: lane 1, Protein $IIIb_1$ (control); lane 2, Protein $IIIb_1$ (1A); lane 3, Protein $IIIb_2$ and $IIIb_3$ (N1A); lane 4, Protein $IIIb_1$ and $IIIb_2$ (N1A); lane 5, Protein $IIIb_2$ and $IIIb_3$ (N1A).

TABLE 3

DISTRIBUTION OF PROTEIN IIIb VARIANTS AMONG CHRONIC ALCOHOLICS AND AGE-MATCHED CONTROLS

	$IIIb_1$	$IIIb_1$ + $IIIb_2$	$IIIb_2$	$IIIb_1$ + $IIIb_3$	$IIIb_2$ + $IIIb_3$	$IIIb_3$	Total
Controls	15	1	—	1	—	—	17
Intoxicated alcoholics	13	2	—	1	—	1	17
Non-intoxicated alcoholics	—	5	2	3	1	1	12

TABLE 4

DISTRIBUTION OF PROTEIN IIIb VARIANTS AMONG VARIOUS CLINICAL GROUPS

	$IIIb_1$	$IIIb_1$ + $IIIb_2$	$IIIb_2$	$IIIb_1$ + $IIIb_3$	$IIIb_2$ + $IIIb_3$	$IIIb_3$	Total
Controls	6	4	—	—	1	—	11
SDAT	5	4	—	3	1	—	13
MID	4	2	—	2	1	—	9
Parkinsonian	—	2	—	—	—	1	3
Dementia NUD	—	—	1	—	—	1	2

studies, the only form of Protein IIIb observed was variant $IIIb_1$ (i.e., only 2 of 17 controls showed the slower migrating variants: $IIIb_2$ and $IIIb_3$). In contrast, all (12/12) non-intoxicated alcoholics showed one or both of the slower migrating forms of Protein IIIb (Table 3). Peptide maps of Protein IIIa showed an analogous distribution of the variants (data not shown).

In a second group of studies, of 27 patients who had suffered from some form of dementia, variant $IIIb_2$ and/or variant $IIIb_3$ was observed in 8 out of 13 SDAT patients, in 5 out of 9 MID patients, in 3 out of 3 Parkinson's disease patients, and in both patients with undefined dementia. Five out of the 11 age matched controls showed variant $IIIb_2$ and/or $IIIb_3$ (Table 4).

The variants of Proteins IIIa and IIIb did not correlate with either cause of death, nutritional state, sex, brain weight, or post-mortem time in any of the groups investigated.

DISCUSSION

Proteins IIIa and IIIb, neuronal phosphoproteins found throughout the brain in association with synaptic vesicles, were studied in normal and in various neuropathological conditions. We have found that the human brain, in contrast to the rat brain, has three variants of these proteins, observed by virtue of differences in their electrophoretic mobilities on SDS-polyacrylamide gels. The variants observed were identical for all brain regions of any given individual. In addition the variants could be detected in material prepared using a variety of different homogenization conditions, suggesting that the appearance of the variants was not dependent upon a particular *in vitro* manipulation. It is also interesting that none of the other approximately 70 phosphoproteins in the brain, so far identified and reported by Walaas *et al.* [14,15], seemed to be altered. One obvious candidate for a similar effect would have been Synapsin I, which has substantial homology with Protein III. However, no change was observed in the mobility of Synapsin I.

The predominant variant of Protein III seen in control human brains comigrated with the rat form of Protein III, while the more slowly migrating variants of Protein III were the predominant forms seen in all non-intoxicated alcoholics. Unfortunately the two sets of control subjects had quite different distributions of the variants. Only 12% of the controls for the study of chronic alcoholics had one of the slower migrating variants of Protein III while 45% of the controls for the dementia study had at least one of these variants. Therefore it would be premature to draw any conclusions about the significance of the observed distribution of the variant forms of Protein III in the various neuropathological conditions examined.

The biochemical explanation for the appearance of the variants of Protein III in the human brain is unclear. It would be desirable to find an experimental model which would reproduce the slowly migrating variants seen in human post-mortem tissue. Since these more slowly migrating variants were consistently seen in non-intoxicated alcoholics, we have examined brain tissue obtained from several rodent models for alcoholism and withdrawal, but have not been able to detect the slower migrating forms (data not shown).

In conclusion, by studying Protein III, a neuronal phosphoprotein, in normal and in several neuropathological conditions, we have been able to identify three variants of the protein. Further investigation of the biochemical basis for the heterogeneity of Protein III in human brain is under way. The fact that the heterogeneity is preserved in a smaller peptide fragment, as shown by one-dimensional peptide mapping, should facilitate these studies.

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